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This past three years of research conducted with support from the Air Force Office of Scientific Research has been directed towards evaluating the use of exhaled breath to estimate the actual exposure to xenobiotics in the work place and the environment. In addition, research has focused on identifying endogenously produced molecules in exhaled breath that serve as sentinel biomarkers of tissue injury and disease. Exhaled breath is composed of many molecules in a gaseous matrix consisting of oxygen, nitrogen, water vapor, carbon dioxide and the inert gases. Endogenously produced molecules are present in concentrations that are less than 100 parts per billon (v/v) whereas the concentrations of exogenous molecules are dependent upon the actual exposrue concentration. However, the composition of exhaled breath can change throughout the normal expiratory cycle and includes molecules that arise from the alveolar membrane junction, conducting airway, mouth and sinuses. Research conducted during this period has been focused on many aspects of breath analysis since the overall goal of this proejct was to collect and analyze representative breath samples from laboratory animals and human subjects.

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FINAL TECHNICAL REPORT

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Summary Abstract

This past three years of research conducted with support from the Air Force Office of Scientific Research has been directed towards evaluating the use of exhaled breath to estimate the actual exposure to xenobiotics in the work place and the environment. In addition, research has focused on identifying endogenously produced molecules in exhaled breath that serve as sentinel biomarkers of tissue injury and disease. Exhaled breath is composed of many molecules in a gaseous matrix consisting of oxygen, nitrogen, water vapor, carbon dioxide and the inert gases. Endogenously produced molecules are present in concentrations that are less than 100 parts per billion (v/v) whereas the concentrations of exogenous molecules are dependent upon the actual exposure concentration. However, the composition of exhaled breath can change throughout the normal expiratory cycle and includes molecules that arise from the alveolar membrane junction, conducting airway, mouth and sinuses.

Research conducted during this period has been focused on many aspects of breath analysis since the overall goal of this project was to collect and analyze representative breath samples from laboratory animals and human subjects. It has been established that repeatable breath samples can be collected from spontaneously breathing human subjects providing that human subject defined tidal volumes and frequencies are used. The collection of breath using two methods, i.e., inert gas sampling bags and collection on the surfaces of well-defined adsorbents contained in glass tubes, has been examined. This latter method of breath sampling was developed in order to transport breath samples by commercial carriers. Since the concentrations of the molecules of interest in breath are low, analytical methods have been developed that are able to concentrate, separate and analyze breath biomarkers using either method of breath collection. Methods have been developed whereby contaminant molecules can be removed from inspiratory air to prevent interference with subsequent breath analysis. These studies were necessary in order to avoid the use of compressed gases in field studies.

Analytical methods that can be used to estimate actual exposure to tetrachloroethylene (trichloroethylene) and the jet fuel JP-8 have been developed. Additionally analytical methods have been developed that can identify and quantify significant numbers of molecules in breath that may be used as biomarkers of tissue injury and disease. The concentrations of various novel biomarkers have been shown to change from normal ranges when abnormal physiology occurs in laboratory rats or human subjects.

It is well-known that a common effect of exposure to environmental or occupational pollutants is the cellular generation of oxygen free radicals that lead to cellular injury and tissue dysfunction. It has been established that breath ethane can be used to determine oxidative stress status of human subjects and laboratory rodents non-invasively. Moreover, it has been demonstrated that breath ethane, a biomarker of oxygen radical-mediated lipid peroxidation, increases when human subjects are exposed to the toxic xenobiotics in cigarette smoke. The effects of controlled diets on the oxidative stress status of human subjects and laboratory rodents have been examined and it has been demonstrated that diets rich in antioxidants, such as those found in fruits and vegetables, can reduce oxidant stress in human subjects.

Risk factors for cardiovascular disease have been causally linked by epidemiological studies to increased levels of environmental or occupational pollutants. It has been suggested that breath isoprene, an elimination product in the biosynthesis of cholesterol, dolichol, ubiquinone and prenylated proteins, may be used to identify human subjects at risk for cardiovascular disease. This link has been examined and it has been determined that human subjects with a variety of hyperlipidemic disorders have elevated breath isoprene.

Hepatic dysfunction has also been linked to exposure to increased levels of environmental or occupational pollutants, particularly organic solvents. Therefore, human subjects with chronic liver diseases have been studied and these studies have resulted in the discovery of novel breath biomarkers that can be used to detect and stage liver disease in human subjects. A provisional patent application reporting this discovery has been submitted.

Since renal dysfunction has also been linked to increased exposure to organic molecules, human subjects with chronic renal diseases have been studied. Preliminary studies have resulted in the discovery of novel breath biomarkers that can be used to detect renal disease in human subjects. This discovery will be validated with a larger number of human subjects prior to the submission of an additional patent application.

Publications / Presentations

Risby, T.H., Burdick, J.F., Foster, W.M., Sehnert, S.S., Breysee, P.N., Rohde C.A., and Mitchell C.S. Human Breath Test for Detection of Early Tetrachloroethylene Hepatotoxicity. **Proceedings of the Air Force Office of Scientific Research Review**, Fairborne Ohio, 1995.

Risby, T.H., Burdick, J.F., Foster, W.M., Sehnert, S.S., Breysee, P.N., Rohde C.A., and Mitchell C.S. Human Breath Test for Detection of Early Tetrachloroethylene Hepatotoxicity. **Proceedings of the Air Force Office of Scientific Research Review**, Fairborne Ohio, 1996.

Miller, E.R. Appel, L.J. Jiang, L. and Risby, T.H. The impact of cigarette smoking on measures of oxidative damage. **Circul**, 94, I143, (1996).

Miller, E.R. Appel, L.J. Jiang, L. and Risby, T.H. The association of cigarette smoking and lipid peroxidation in a controlled feeding study. **Circul**. 96, 1097-1101, (1997).

Risby, T.H., Burdick, J.F., Foster, W.M., Sehnert, S.S., Breysee, P.N., Rohde C.A., and Mitchell C.S. Human Breath Test for Detection of Early Tetrachloroethylene Hepatotoxicity. **Proceedings of the Air Force Office of Scientific Research Review**, Fairborne Ohio, 1997.

Miller, E.R. Appel, L.J. Jiang, L. and Risby, T.H. The effect of dietary patterns on measures of lipid peroxidation: results from a randomized clinical trial. **Circul**. In press, (1998).

Risby, T.H, Jiang,L., Stoll, S. Ingram, D. Spangler, E. Heim, J. Cutler, R., Roth, G.S. and Rifkind, J.M. Breath Ethane as a Marker of Reactive Oxygen Species Generated During Manipulation of Diet and Oxygen Tension in Female Fischer 344 Rats. Submitted **J.Appl Physiol, (1998).**

Risby, T.H., Burdick, J.F., Foster, W.M., Sehnert, S.S., Breysee, P.N., Rohde C.A., and Mitchell C.S. Prediction of cellular dysfunction from exposure to JP-8. **International Conference on the Environmental Health and Safety of Jet Fuel**. San Antonio TX., 1998.

Risby T.H., Sehnert S.S., Jiang L., and Burdick J.F. Breath Biomarkers for Detection and Staging Human Liver Diseases. To be submitted to **New England J. Medicine**

Jiang, L. Gao, Z.H., Sehnert, S.S., Burdick, J.F., Rohde, C.A., Kwiterovich, P.O. and Risby, T.H. Breath Isoprene in Human Subjects with Normal and Abnormal Blood Lipid Profiles. In preparation

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Coupling Activities

As a result of our funded research in the field of breath analysis we have developed active collaboration with the following investigators. These collaborations have been directed towards different aspects of our research program.

Collaborations aimed at the development of commercial systems for collection and analysis of breath biomarkers:

William Betz, Ph.D.
Supelco Corporation

Alan Ganz, Ph.D.
Perkin Elmer Corporation

John Connolly, Ph.D.
Sarnoff Corporation

The following collaborators has been supplied with the cryogenic and thermal desorption system developed during this research:

Gwynne Jones, M.D.
University of Ottawa College of Medicine

Garry Handelman, Ph.D.
Tufts University College of Medicine

George Perry, Ph.D.
Case Western Reserve University, College of Medicine

John Windsor, M.D.
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We have collaborated with the following researchers by performing breath analyses in mutually interesting research projects:

Joseph Rifkind, Ph.D.
National Institute of Aging

Edgar Miller, M.D.
Johns Hopkins University School of Medicine

Andrew Warren, M.D., D. Phil.
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We have coordinated our research on the effects of the jet fuel JP-8 through active collaboration with the following investigators funded by Air Force Office of Scientific Research:

Mark Witten, Ph.D.
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University of Arizona College of Medicine

David Harris, Ph.D.
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Frank Siegel, Ph.D.
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Steven Kornguth, Ph.D.
University of Wisconsin College of Medicine

Frank Witzmann, Ph.D.
Indiana University

Discoveries, Inventions, Patent Disclosures, and Specific Applications

A provisional patent application entitled, "Breath Biomarkers for Detection and Staging Human Liver Diseases" was filed with the United States Patent Office in Crystal City, Virginia on August 31, 1998. This application originates from work performed during this Air Force Office of Scientific Research-sponsored research. Because of the laws and regulations involving "public disclosure" in the patent process, we have been unable to submit manuscripts reporting this research for publication. A manuscript has been prepared and will be submitted to the New England Journal of Medicine. Plans to commercialize this patent application are underway and discussions are on-going with various instrument companies and venture capital companies.

Research Accomplishments

Objectives:

The original specific aims of this study were to: 1.) Identify and quantify breath sulfur- and nitrogen-containing biomarkers of liver dysfunction; 2.) Generate a library of these breath biomarkers for various liver diseases; 3.) Investigate whether increased free radical generation, as measured by levels of breath ethane, occurs as a result of a 4 hour exposure to occupational levels of tetrachloroethylene; 4.) Investigate whether CNS toxicity, as measured by airway occlusion pressure, occurs as a result of a 4 hour exposure to occupational levels of tetrachloroethylene; and 5.) Demonstrate that breath biomarkers can be used to identify people who have liver dysfunction as a result of occupational exposure to tetrachloroethylene.

When the research plan was originally submitted, we had a commitment from International Fabricare Institute (IFI) in Washington, DC to allow us to study their association's drycleaners. Some drycleaners are occupationally exposed to tetrachloroethylene. In the interim, this commitment evaporated since employers were afraid of potential litigation. Subsequently, we had discussions with industrial hygienists at the Department of Defense who were responsible for out-sourcing drycleaning for the federal government, however these commercial drycleaners also refused permission to sample their workers. This problem was discussed with the AFOSR Program Manager and other US Air Force personnel and during these discussions we were informed that the US Air Force was no longer interested in occupational exposure to tetrachloroethylene. Furthermore, it was suggested that we should initiate studies aimed at estimating potential health effects resulting from exposure to JP-8.

Accomplishments:

In the past three years research has been conducted in a number of areas: breath collection; breath collection media; breath analysis; breath biomarkers of: oxidative stress status, cholesterol biosynthesis, hepatic dysfunction, renal dysfunction, and exposure assessment; and the evaluation of tests that could be used to assess changes in neuropsychological function as a result of exposure to JP-8.

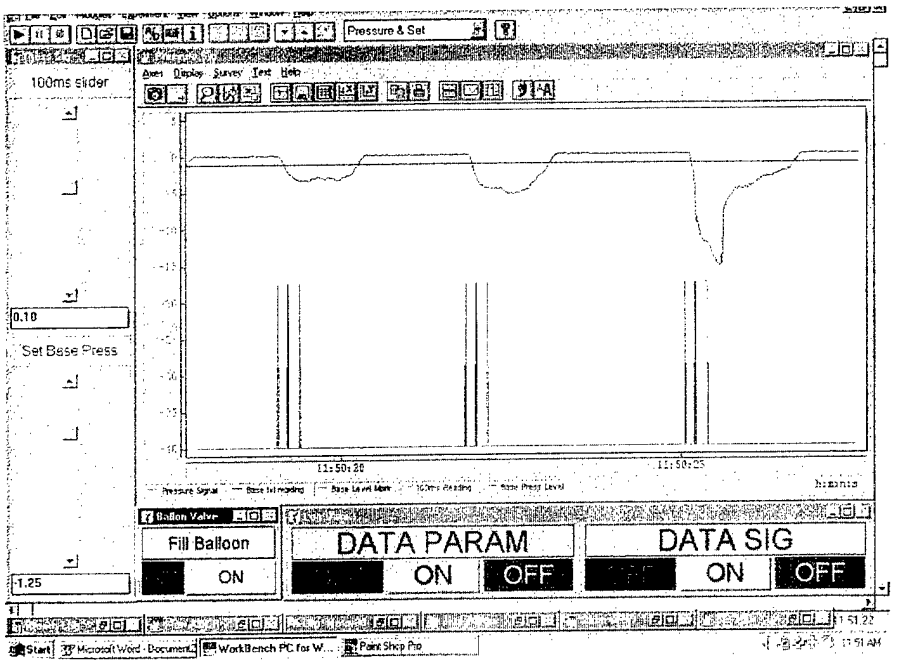
Breath Collection

a. Laboratory Rodents:

A specially designed flow-through glass chamber was constructed that allows the exhaled breath of a rat to be sampled in the outflow gas from the chamber. In order to avoid stressing the rat the concentration of carbon dioxide in the outflow gas of the chamber is monitored continuously. Additionally, turbulent air flow through the chamber is maintained to ensure that the gas collected at the outflow is representative. In order to use this chamber for collection of respiratory, gas the rat is initially equilibrated to hydrocarbon free air by high flow of gas through the chamber. After this period of equilibration (generally 50 volume changes) the air flow rate is reduced and the outflow gas from the chamber is collected for measurement. Typically, during sample collection the concentration of carbon dioxide in the outflow gas is maintained below 7 Torr. The ability to collect rat breath using this chamber has been demonstrated and, in addition, physiological measurements of oxygen consumption and carbon dioxide production have agreed with published values.

b. Human Subjects:

Various approaches to collect breath from spontaneously breathing human subjects have been examined in order to obtain representative, reproducible breath samples. The techniques of breath holding and collection of end-tidal breath samples were evaluated without success. On the basis of this research it has been established that the optimum method to collect breath is from an upright seated subject who is receiving visual aids from a computer screen that displays their breathing frequencies, tidal volumes and carbon dioxide profiles. A respiratory workbench that is microprocessor-controlled and can be changed to meet the comfortable respiratory requirements for each study subject has been developed. Parameters such as the respiration rate, tidal volume, end-tidal carbon dioxide concentration, minute ventilation, and average carbon dioxide concentration are recorded continuously during breath collection. The ratio of average carbon dioxide concentration to end-tidal carbon dioxide concentration is used to evaluate the anatomic dead-space and evaluate the breathing of the study subject. If this ratio falls below 50% the breath collection is repeated. This system uses commercially available modules. Briefly, the study subject inspires and expires through a disposable mouthpiece and in-line biological filter into the mouth port of a non-rebreathing valve. The inspiratory port of this non-rebreathing valve contains a pressure sensor that is used to trigger the computer program to each breath cycle. The inspiratory port also includes a balloon valve that allows the inspiratory port to be occluded under computer control. During occlusion the drop in pressure is measured by the pressure sensor. Occlusion pressure is simply the pressure measured in the airway of a spontaneously breathing subject after the airway has been occluded just at end-expiration. The occlusion pressure has been widely used to evaluate neuronal output of the respiratory centers of the brain. The inspiratory air is filtered with a disposable charcoal respiratory cartridge. This approach was found to produce a minimum back pressure on the inspiratory port of the non-rebreathing valve. It has been established that these respiratory cartridges can remove airborne contaminants from the inspired air for at least 30 minutes although a new cartridge is used for each study subject. Study subjects have been found to require approximately 5 minutes of training to use this system and during this training time an effective washout of exogenous pollutants from ambient air is achieved. Spontaneous, relaxed breathing is sampled for a period of 1 or 3 minutes depending upon the method of breath collection. The coefficient of variation for breath samples has been found to be less than 5%. Examples of the computer screens for pressure, flow and carbon dioxide are shown on the subsequent page. These are the displays that study subjects view as aids to control their breathing patterns. This technique represents a significant advance in the science of precision breath analysis.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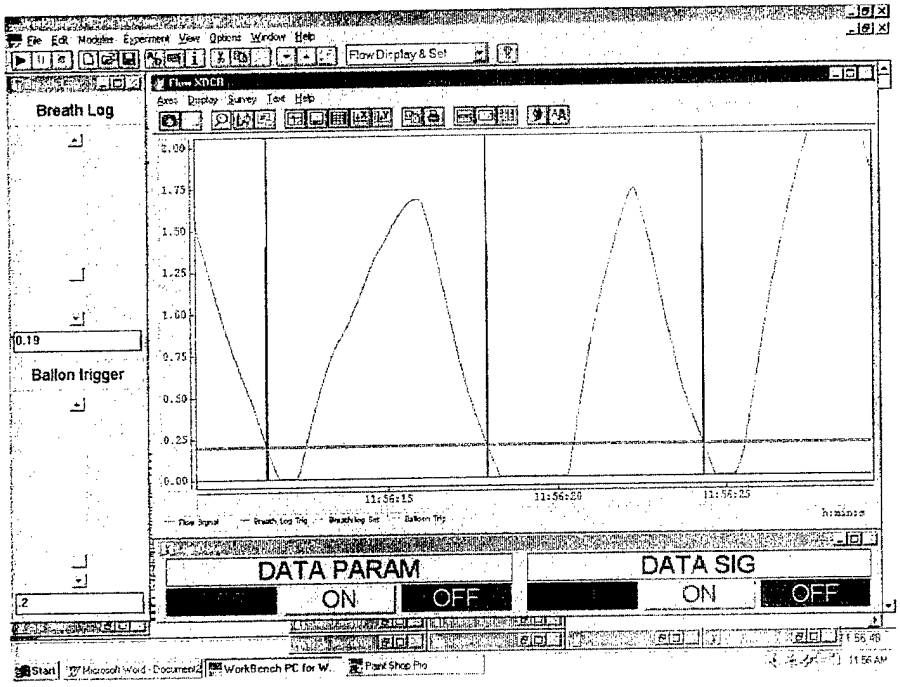
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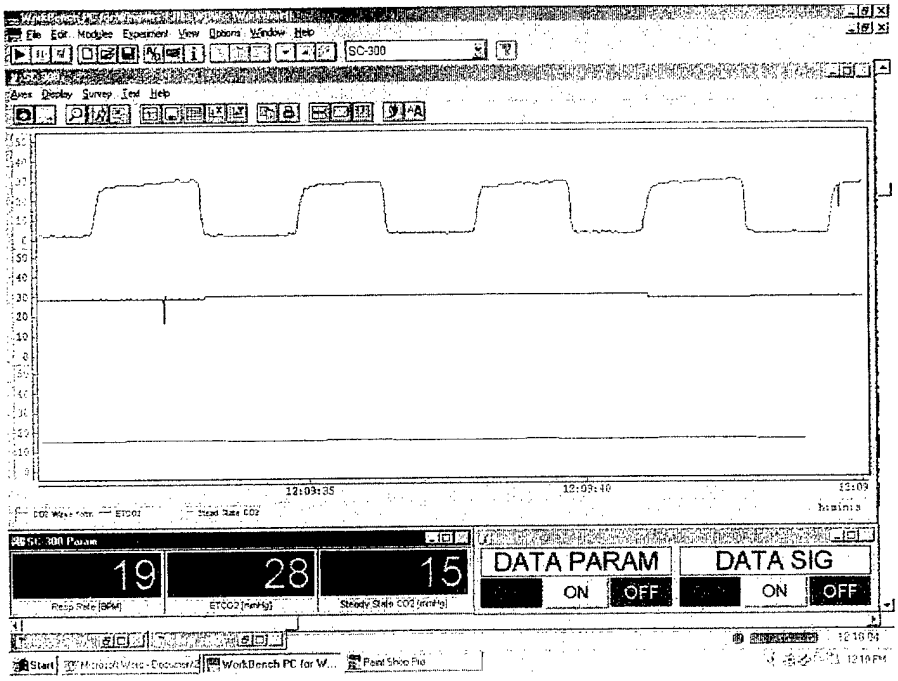
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Breath Collection Media

Two methods for the collection of breath samples have been examined during the course of this research. Each method has distinct advantages.

a. Collection in Inert Gas Sampling Bags:

The collection of breath over a one minute period of time in specially designed inert gas sampling bags was the approach investigated initially. This approach was preferred since it allows triplicate analyses to be performed on each sample. It was established that the concentrations of specific breath biomarkers (ethane, isoprene, acetone and carbon dioxide) remained stable in these bags for at least 48 hours after collection. However, the collection of breath in the field, where transport of the collected breath in gas sampling bags by commercial airlines is required, precludes the use of this method of breath collection.

b. Collection on Thermal Desorption Tubes:

Collection of breath samples using commercial multibed adsorbents packed in thermal desorption tubes was then examined since this method of breath collection is ideally suited for field studies. Commercial thermal desorption tubes have unique numbers engraved on the tube that can be used to track each sample. We evaluated a number of adsorbents for their ability to collect the molecules of interest in exhaled breath. The major problems with this method of collection were caused by the high concentrations of water vapor and carbon dioxide that reduced the collection efficiencies. The ideal adsorbent should allow sufficient breath to be sampled so that the signal-to-noise ratio of the analyte molecules is greater than 3:1.

Various non-porous graphitized carbons (Carbotrap Y, Carbopack B and Carbopack X) with surface areas in the range of 24 -240 m^2/g, were examined. These adsorbent are weak and will adsorb all compounds greater than about C_4 or C_5. Additionally various carbon molecular sieves (Carbosieve S-111, Carboxen-1010, Carboxen-1000, and Carboxen -1008) with surface areas in the range of 700 -1200 m^2/g were examined. These strong adsorbents contain different quantities of micro- meso and macro-pores and will adsorb compounds in the range C_1 through C_5. Breath was drawn through the weak adsorbent bed followed by the strong adsorbent bed packed sequentially in the same tube. Differing amounts of these adsorbent beds were examined. At the time of this report, we feel that the optimum packing is a packing consisting of 50% Carbopack X and 50% Carboxen 1010. This design of

adsorbent bed has been shown to collect all the molecules of interest and allow subsequent thermally desorption and analysis by capillary gas chromatography.

Other variables examined were the sampling rate and the volume sampled. Breath is drawn through the thermal desorption tubes using a battery-operated commercial two-tube sampler. This sampler consists of a rechargeable battery, vacuum pump, programmable controller that controls the sampling time. The flow at each of the two-tubes can be controlled independently. On the basis of studies to date the optimum flow rate has been found to in the range of 30 mL/min with an optimum sampling time of three minutes. Increasing the flow rate and reducing the sampling time decreased the collection efficiency. Duplicate samples are obtained.

After the sample has been collected, the thermal desorption tube is capped with Swagelok fittings and stored until it is analyzed. Research has shown that breath and air samples are stable in thermal desorption tubes for at least a year, although all samples are analyzed as soon as possible.

Breath analysis

a. Cryogenic Concentration and Thermal Desorption :

Extensive studies have been performed on the concentration of exhaled breath and the subsequent analysis by gas chromatography. As a result of these studies, a simple approach has been developed that allows gaseous samples to be sorbed onto Tenax TA at -117°C and then rapidly thermally desorbed directly onto the head of a fused silica wide-bore capillary gas chromatographic column. The concentration of the gaseous samples is as follows: A stainless steel wide bore capillary tube (15 cm, 1.65 mm o.d.; 1.19 mm i.d.) packed with 2,6-diphenyl-p -phenylene oxide (60-80 mesh Tenax TA®) connected to a six-port stainless steel gas sampling valve (1.59 mm inlets) in place of the standard gas sampling loop. The length of the Tenax packing is 10 cm and the packing is retained on either side with silanized glass wool. This collection tube is submerged in an ethanol/liquid nitrogen slush bath (-117°C). The collection tube is maintained in the liquid nitrogen slush bath for ten minutes in order to allow the adsorbent to equilibrate to -117°C, and then 30 mL of collected breath is drawn through the adsorbent using a gas-tight syringe. A disposable trap containing Ascarite is placed between the gas sampling bag and the collection tube to remove carbon dioxide from the breath sample. After the breath had been sampled, the liquid nitrogen slush bath is replaced with a specially designed heating block maintained at 160°C and the concentrated gas sample is injected immediately onto the gas

chromatographic column by rotation of the gas sampling valve. The valve is rotated back to its fill position after 45 seconds and the collection tube is flushed with nitrogen to clean it prior to the next sample collection. The collection tube and adsorbent reach 160°C within 30 seconds. The use of a reduced temperature has been found to increase the distribution constant for the sorption of low boiling point gases onto the surface of Tenax TA. Thermal desorption at 160° C has been sufficient to quantitatively desorbed the breath compounds identified to date. Methane and ethane are quantitatively sorbed at this temperature without trapping the major constituents, nitrogen or oxygen. The break-through volumes of breath analyte molecules identified to date have been investigated and at -117°C, all these molecules can be collected with approximately 100% efficiency using volumes of breath of up to 200 mL. Similarly, the thermal desorption temperature (160°C) has been shown to quantitatively desorb the molecules of interest without significant tailing of the solute peaks. The concentration system has been validated by comparing the results of the analyses of the analyte molecules identified-to-date using regular gas sampling loops connected to the gas sampling valve and more concentrated gas samples to the results of the analysis of the same analyte molecules contained in diluted gas samples using the sorbent packed sampling loop with cryogenic cooling and thermal desorption. Separation of the components of breath were performed using 60 m fused silica capillary columns wall-coated with 5μm dimethyl silicone. The temperature protocol is as follows: Hold at 25°C for five minutes, 25-200 at 10°C/min and hold at 200°C for five minutes. Linear gas velocities of 25 cm/sec at 25°C is used for all chromatographs. Gas chromatographs equipped with flame ionization, a thermionic, a flame photometric or electron impact mass spectrometric detectors were used in these studies. These detectors have been selected for use in the proposed studies in order to quantify the analyte molecules of interest. The flame ionization detector is a non-selective detector and responds to most compounds. The thermionic detector responds to nitrogen containing compounds and the flame photometric detector responds to compounds containing sulfur atoms. Electron impact mass spectrometric detection was used to identify and confirm the identity of unknown compounds. Analytical methods were calibrated daily by injection of a standard gas mixtures of the requisite analyte molecules using standard gas sampling loops. We have demonstrated that the limit of quantification using 30 mL of exhaled breath is sufficiently sensitive for the desired analysis, i.e., 0.1 ppb. The rates of exhalation of the analyte molecules were expressed in units of ppb or pmol/min-kg corrected to the end tidal concentration of 40 Torr of carbon dioxide.

b. Two Stage Thermal Desorption:

Analysis of breath samples that have been adsorbed on the multi-bed adsorbent packed in thermal desorption tubes were performed using commercial two-stage thermal desorption systems that are coupled either to a gas chromatograph with parallel flame ionization, and flame photometric detectors or to a gas chromatograph with parallel thermionic and electron impact mass spectrometric detectors.

The automated two-stage thermal desorption system can handle up to 50 samples sequentially. The process by which the gas sample that was collected in the field is analyzed by capillary gas chromatography is as follows: the long-term storage Swagelok caps that were placed on each thermal desorption tube after the sample had been collected are removed and replaced by polytetrafluoroethylene end caps. The resulting thermal desorption tubes are inserted into 50 tube carousel and the analysis is performed automatically. The two-stage thermal desorption system takes each sample tube in turn, and uncaps it in the carrier gas stream. It will perform a leak test to ensure that the tube is sealed correctly and then heats the tube to 200°C for five minutes with the carrier gas flowing through the multibed adsorbents. Thermal desorption is performed with the flow in the opposite direction to the flow used originally to adsorb the sample. This reverse flow ensures quantitative thermal desorption of the adsorbed molecules. The thermally desorbed molecules are concentrated in a low thermal mass cold trap (-30°C) containing small amounts of the same carbonaceous adsorbents. The cold trap is heated rapidly to at a rate of 40°C/s to 200 °C and the thermally desorbed molecules are transferred through a heated fused silica transfer line to the head of a fused silica capillary column.

Separation of the components of breath are performed using capillary gas chromatography using 0.32 mm. 60 m fused silica capillary columns wall-coated with 5μm dimethyl silicone. The separation protocol is as follows: isothermal at 35°C for ten minutes, 35-210 at 5°C/min, isothermal at 210°C for ten minutes. A linear gas velocity of 25 cm/sec at 25°C is used. The available detectors enable all the analyte molecules of interest to be quantified. These two-stage thermal desorption capillary gas chromatographic systems are microprocessor controlled and data are stored digitally.

Breath Biomarkers

During the course of this research, known breath biomarkers have been examined in novel studies in human subjects and laboratory rodents and new biomarkers have been identified in human subjects presenting with chronic diseases.

a. Oxidative Stress Status

The effects of nutritional status and life styles on oxidative stress status was investigated since these factors may be significant factors in assessing the effects of exposure to airborne pollutants. These factors were examined in a blinded case-controlled feeding study with normal human volunteers. The protocol for this study was to feed a typical American diet to a group of volunteers for three weeks. After three weeks these volunteers were then fed one of the following three diets for an additional eight weeks: 1.) the same diet; 2.) a diet with increased amounts of fruits and vegetables or; 3.) a diet with increased fruit and vegetables and reduced fats. The selection of diet was blinded to both the volunteer and the investigators. Various clinical parameters including breath samples were collected at the end of the initial run-in and at the end of the study. The following summarizes some of the results of this study:

- levels of breath ethane were statistically higher in smokers than non-smokers demonstrating the pro-oxidant effects of the xenobiotic cigarette smoke;
- level of breath ethane could be correlated to the time between smoking a cigarette and the time of breath collection;
- increasing the amounts of fruits and vegetables statistically reduced the levels of breath ethane in the same individual;
- all individuals fed on diets with increased fruits and vegetables had statistically lower breath ethane as compared to those fed on a diet with lower amounts of fruits and vegetables.

Similar studies were conducted with laboratory rats. These studies were performed to check the use of the flow-through chamber as a means to collect rat breath. Dietary restriction without malnutrition is a well-established approach to alter the rate of aging in laboratory animals. Additionally, an accepted theory of normal aging involves the age-related changes in oxidative stress. Therefore, we have investigated whether breath ethane could be used to quantify oxidative stress status in aged rats that have been maintained on controlled diets. For these studies 22 month old female Fischer 344 rats that have been maintained beginning at six weeks of age on either restricted or *ad libitum* diets were examined. Each rat was placed in a flow-through glass chamber and breath ethane was measured at the outlet of the chamber. *Ad libitum* fed rats were shown to have levels of breath ethane that were statistically significant greater than the levels of breath ethane for those rats that had

been dietary restricted, demonstrating that breath from rats can be collected and quantified and used to evaluate theories of aging. Additionally, these studies established that the concentration of breath biomarkers should be corrected for carbon dioxide production rate. This correction is superior to correcting based upon body weight for animals with widely different masses of body fat. Increased amounts of body fat do not change metabolic rates.

b. Cholesterol Biosynthesis

The utility of breath isoprene to monitor endogenous production of cholesterol was tested in a group of patients presenting with a variety of hyperlipidemic disorders (n=82) who attended a lipid clinic at The Johns Hopkins Hospital. The levels of breath isoprene in this group of patients were compared to levels of isoprene determined in breath collected from a group of normal volunteers (n=109). The following is a summary of our results to date:

- levels of breath isoprene show a quadratic dependence with age in all human subjects *i.e.*, they increase from below detectable at birth, to detectable levels at about six months, reach a plateau in the mid-forties and decline after 50 years of age;
- males had higher levels of breath isoprene compared to age-matched females, this difference was less pronounced after 50 years of age;
- patients judged to have familial combined hyperlipidemia (Type IIA) had statistically higher levels of breath isoprene compared to aged-matched patients judged to have other hyperlipidemic disorders;
- patients judged to have familial hypercholesterolemia (Type IIB) had statistically higher levels of breath isoprene compared to aged-matched normals;
- breath isoprene can be used to monitor efficacy of lipid-lowering pharmacological agents.

c. Hepatic Dysfunction

Our results to date on the use of breath analysis to detect abnormal liver function are based upon the collection of breath and blood samples from normal human volunteers (n=112, 74 males and 48 females) and human subjects with known liver disease who are the "disease" control for this study (96 subjects, 59 males and 37 females alcoholic cirrhosis (n=20), alpha-1-antitrypsin deficiency (n=1), autoimmune cirrhosis (n=7), cryptogenic cirrhosis (n=15), cystic fibrosis (n=1), fulminant hepatitis (n=4), hepatitis B (n=5), hepatitis C (n=21), primary biliary cirrhosis (n=6), sclerosing

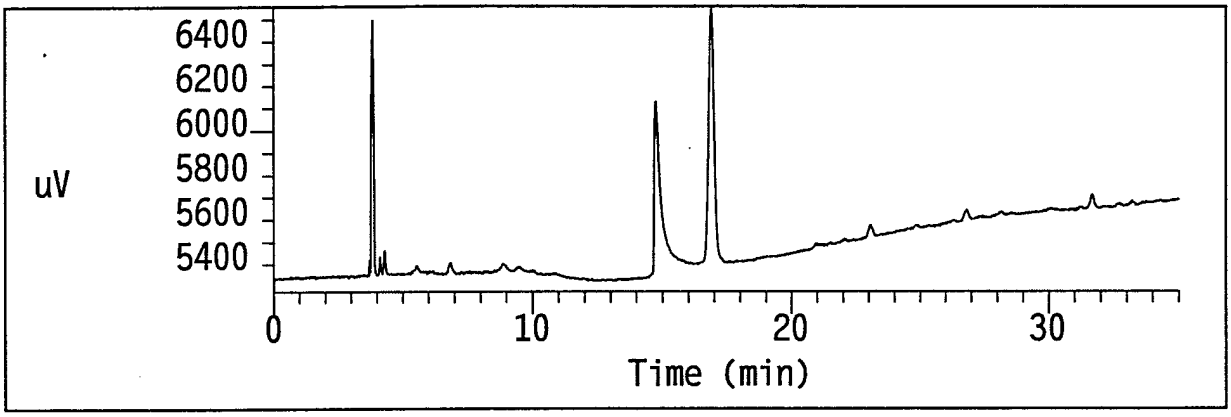
cholangitis (n=14), steatohepatitis (n=4), and biliary obstruction (n=1)). Additionally, the stage of the liver disease in each patient was graded using standard clinical criteria as follows: early-, mid-, and end-stage liver disease. Various statistical methods (ANOVA, multiple regression analysis, logististic regression analysis, Student's t test, Scheffe test, Bonferroni multiple comparison test, nonparametric tests, kruskal-Wallis test) were used to examine the results of blood and breath tests to identify those parameters that distinguished between normal human subjects and patients with liver disease. Study subjects with liver diseases were separated into two groups: those whose diseases originated in the hepatocytes (alcoholic cirrhosis, alpha-1-antitrypsin deficiency, autoimmune cirrhosis, cryptogenic cirrhosis, fulminant hepatitis, hepatitis B, and hepatitis C, and steatohepatitis) and whose diseases originated in the bile duct (cystic fibrosis, primary biliary cirrhosis, sclerosing cholangitis, and biliary obstruction). All the clinical and breath data were found to be not normally distributed and, therefore, only non-parametric tests could be used to investigate the relationships between disease status and breath biomarkers using the raw data. However, these data were normally distributed if they were logarithmically transformed. Therefore, data analysis was performed using raw and transformed data. Briefly, the results of our studies are as follows:

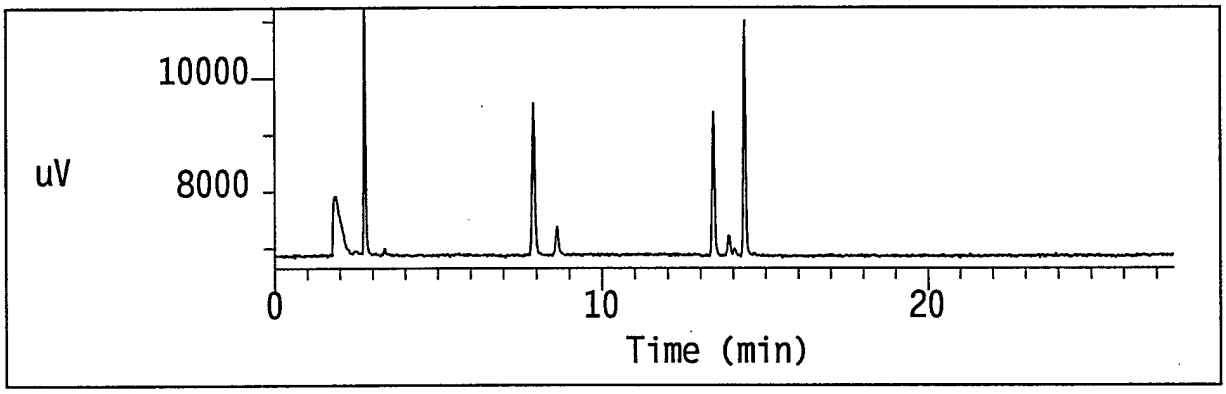
- breath carbonyl sulfide can be used to distinguish: between normal human subjects and patients with liver disease ($p<0.00001$); between normal human subjects and patients with hepatocellular disease ($p<0.00001$); between normal human subjects and patients with diseases of the bile duct ($p<0.00001$);
- breath dimethyl sulfide can be used to distinguish: between normal human subjects and patients with liver disease ($p<0.05$); and between normal human subjects and patients with hepatocellular disease ($p<0.045$);
- breath ethane can be used to distinguish: between normal human subjects and patients with liver disease ($p<0.017$); and between normal human subjects and patients with hepatocellular disease ($p<0.02$);
- breath carbonyl sulfide can be used to stage liver disease ($p<0.00001$);
- breath dimethyl sulfide can be used to stage hepatocellular injury disease ($p<0.01$);
- breath ethane approached can be used to stage hepatocellular injury ($p<0.03$).

The analysis of a typical breath sample collected from a patient with liver disease is shown on the following page.

Analysis of a breath sample from a study subject with liver disease

Top chromatogram is the response of a flame ionization detector
Bottom chromatogram is the response of a flame photometric detector





d. Renal Dysfunction

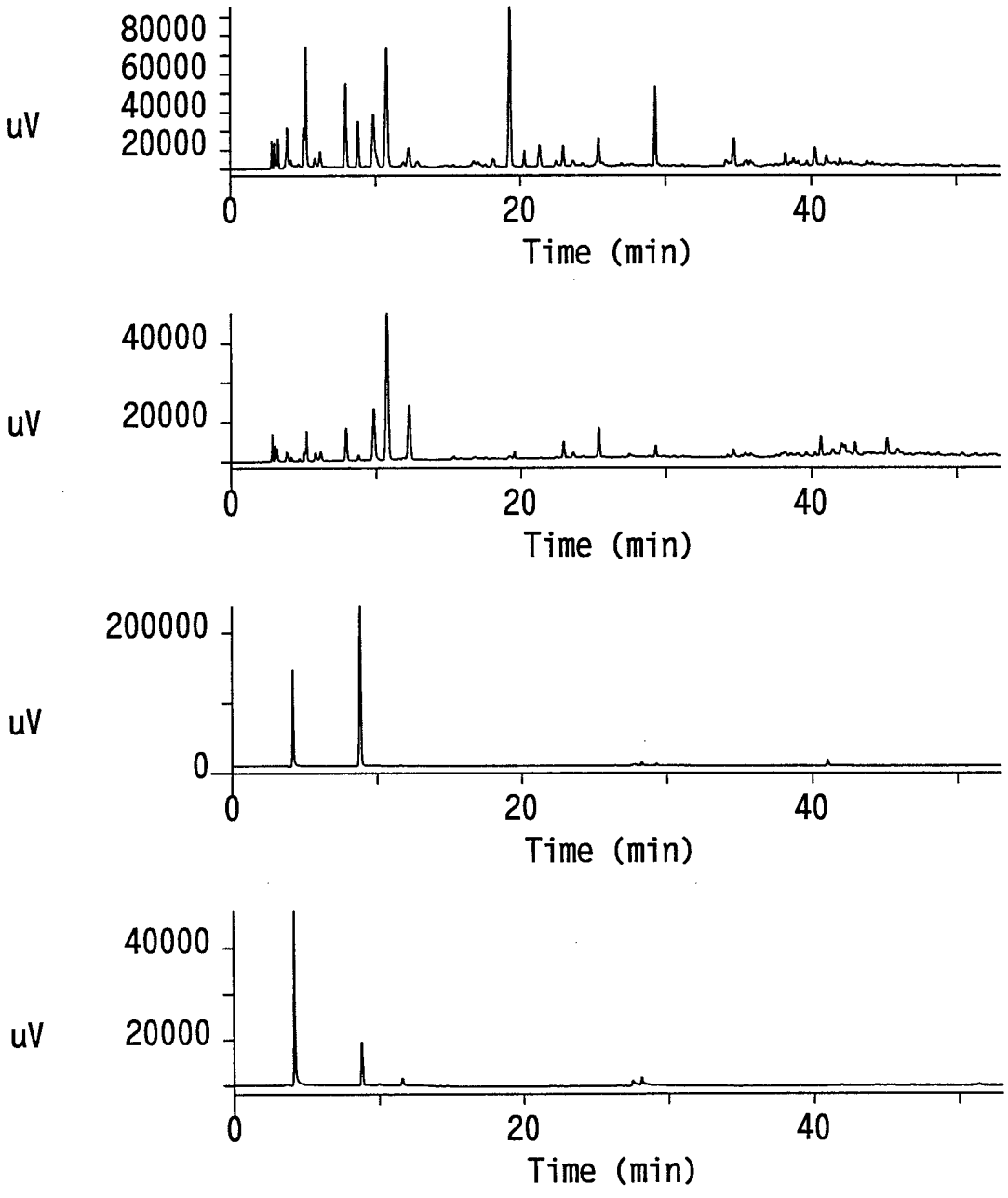
We have performed some preliminary studies aimed at identifying breath biomarkers of kidney dysfunction in humans. For this study we selected a group of 35 patients undergoing dialysis for renal failure. We collected breath in tubes before and after dialysis for each subject, therefore, each patient acts as his/her own control. Based upon preliminary examination it appears that there may be as many as 10 compounds (ketones, amines) present in human breath that decrease in concentration after dialysis. These results are extremely exciting since this is a novel approach for investigating kidney disease and is currently being evaluated in a larger population. Example of the results of the analysis of breath collected from the same patient before and after dialysis is shown on the following page. These breath biomarkers may serve as biomarkers of nephrotoxicity.

e. Exposure Assessment

Methods to assess the concentrations of tetrachloroethylene and trichloroethylene in ambient air were developed based upon collection of air samples in gas sampling bags. These methods were also able to determine the background levels of any of the biomarkers identified in human breath. The limits of quantification for all these molecules were in the range of 0.1 parts per billion based upon 30 mL sampling volume. Only ethane was found to be present in measurable amounts in ambient air (around 2 ppb). The presence of all these molecules were shown to be reduced to below detectable levels when ambient air was passed through the charcoal respiratory cartridges. The use of passive dosimeters to collect ambient air was also examined. Desorption of adsorbed tetrachloroethylene and trichloroethylene by thermal means was found to be irreproducible and solvent extraction with carbon disulfide was found to be superior. Preliminary studies with the commercial thermal desorption tubes have been performed and the same protocol can be used to collect breath samples can be used to collect airborne JP-8. Additionally we have collected the breath from rats that have been exposed to JP-8 at the University of Arizona and based upon studies to date we have determined that JP-8 can be detected in exhaled breath for only about an hour and a half after exposure. These results are currently being confirmed in additional studies and will be important to future studies directed towards occupational exposure to JP-8. Examples of the breath collected from non-exposed and exposed rats are shown on the following page.

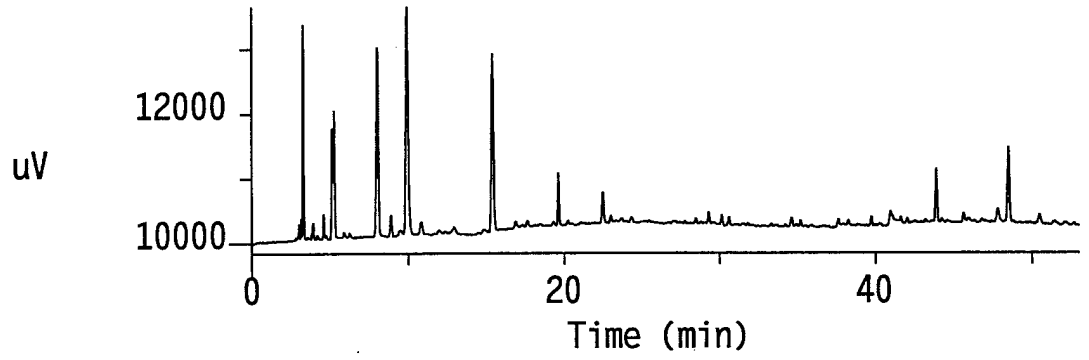
Analysis of breath samples collected from the same study subject before and after dialysis

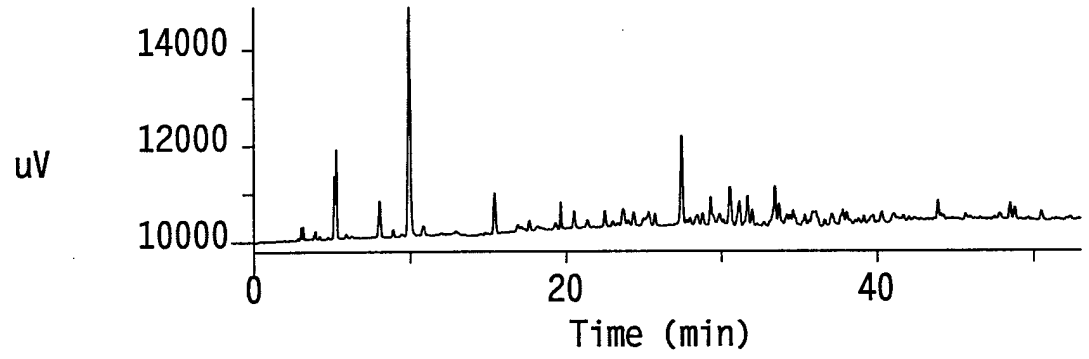
Top two chromatograms are the responses of the flame ionization detector
Bottom two chromatogram are the the responses of the thermionic specific detector

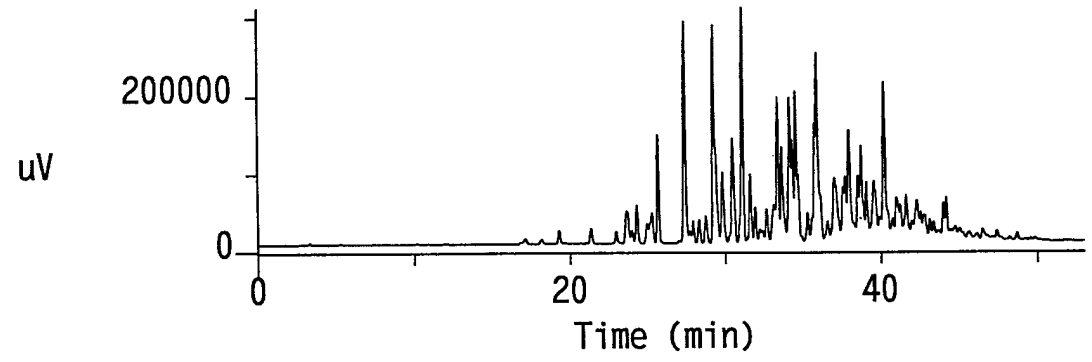


Analysis of breath samples collected from rats

Top chromatogram is the analysis of the breath from a non-exposed rat
Middle chromatogram is the analysis of the breath from a rat exposed to JP-8
Bottom chromatogram is the analysis of the JP-8 aerosol used for exposure (900mg/m^3)







Neuropsychological Function

At the request of Air Force Office of Scientific Research to develop the capability to evaluate changes in neuropsychological functioning as a result of exposure to the jet fuel JP-8. A number of commercially available testing protocols have been examined. The goal of the neuropsychological functioning test is to evaluate whether cognitive functioning of air force personnel is changed as a result of exposure to JP-8. The computer based CogScreen-Aeromedical testing program marketed by Psychological Assessment Resources Inc. has been selected for use in the planned field studies with air force personnel. The program description provided by the supplier states that this test is administered on-screen and is scored automatically. A series of self-contained cognitive tasks assesses deficits or changes in attention, immediate and short-term memory, visual-perceptual functions, sequencing functions, logical problem solving, calculation skills, reaction time, simultaneous information processing abilities and executive functions. Since we wish to avoid the potential problem of test training we plan to test air force personnel after a normal eight hour work period. This will provide neuropyschological tests results after exposure. We will retest each person at least four months later after a period of at least two days of non-exposure to JP-8 at the start of the workday. We will then compare the results of this test to establish whether there is any evidence of changes in cognitive function as a result of exposure to JP-8. Each person will act as his or her own control. This test has a variety of test reports and our results can be compared to a normative sample of airline pilots. The selection of this tests was made in consultation with Dr. Andrew Warren, an attending psychiatrist in the Johns Hopkins University School of Medicine. Dr. Warren has agreed to help evaluate the results of this test.